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Role of Lipase from Community-Associated Methicillin-Resistant *Staphylococcus aureus* Strain USA300 in Hydrolyzing Triglycerides into Growth-Inhibitory Free Fatty Acids

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Part of the human host innate immune response involves the secretion of bactericidal lipids on the skin and delivery of triglycerides into abscesses to control invading pathogens. Two *Staphylococcus aureus* lipases, named SAL1 and SAL2, were identified in the community-associated methicillin-resistant *S. aureus* strain USA300, which, presumably, are produced and function to degrade triglycerides to release free fatty acids. We show that the SAL2 lipase is one of the most abundant proteins secreted by USA300 and is proteolytically processed from the 72-kDa proSAL2 to the 44-kDa mature SAL2 by the metalloprotease aureolysin. We show that spent culture supernatants had lipase activity on both short- and long-chain fatty acid substrates and that deletion of *gehB*, encoding SAL2, resulted in the complete loss of these activities. With the use of gas chromatography-mass spectrometry, we show that SAL2 hydrolyzed trilinolein to linoleic acid, a fatty acid with known antistaphylococcal properties. When added to cultures of USA300, trilinolein and, to a lesser extent, triolein inhibited growth in a SAL2-dependent manner. This effect was shown to be due to the enzymatic activity of SAL2 on these triglycerides, since the catalytically inactive SAL2 Ser412Ala mutant was incapable of hydrolyzing the triglycerides or yielding delayed growth in their presence. Overall, these results reveal that SAL2 hydrolyzes triglycerides of both short- and long-chain fatty acids and that the released free fatty acids have the potential to cause significant delays in growth, depending on the chemical nature of the free fatty acid.

Staphylococcus aureus is a human commensal Gram-positive bacterium found to persistently colonize about 30% of the human population, yet it is also recognized as an opportunistic pathogen, causing infections ranging from mild skin and soft tissue infections to more severe conditions such as necrotizing pneumonia, osteomyelitis, toxic shock syndrome, bacteremia, and infective endocarditis (1, 2). Accordingly, it is not surprising that *S. aureus* produces a large repertoire of virulence factors, including surface-associated proteins and polysaccharides, and numerous toxins, exoenzymes, and immune evasion factors which collectively contribute to the success of this bacterium as a pathogen (1, 3, 4). Antibiotic-resistant strains of *S. aureus*, and especially their rapid spread around the globe, represent a significant threat to the health of the population, as evident from statistics which reveal that the mortality attributed to *S. aureus* infection is now comparable to rates for AIDS, tuberculosis, and viral hepatitis (5–7), and higher mortality is associated with methicillin-resistant *S. aureus* (MRSA) (8).

Strains of MRSA are among the most noted pathogens currently circulating (9), and these are dominated by a particularly hypervirulent strain of community-associated MRSA (CA-MRSA), USA300, which exemplifies the documented ability of CA-MRSA to cause infections in otherwise healthy individuals and efficient person-to-person spread (10–14). Consequently, from its first appearance as a CA-MRSA strain in the community setting in the late 1990s, USA300 has now become the predominant strain of *S. aureus* in both the community and hospital settings across North America (15, 16). An alarming implication of this rapid transcendence and predominance is associated with the fact that approximately 30% of the human population exhibit stable nasal carriage of *S. aureus*, and among those who do exhibit nasal carriage, *S. aureus* is also frequently recovered from the skin of the forearms, hands, and chest, as well as the axillae and perineum (17). Therefore, a significant portion of the population will exhibit asymptomatic carriage of USA300, which is a risk factor for subsequent infection.

Recent studies have indicated that the emergence and rapid geographic spread of USA300 were due, at least in part, to its enhanced ability to persist on human skin, which was attributed to a mobile genetic element that uniquely confers resistance of USA300 to toxic polyamines encountered on the skin, to which all other strains of *S. aureus* are sensitive (18, 19). Nevertheless, *S. aureus* as a species is capable of colonizing humans and must also cope with other innate defense mechanisms of the skin, the foremost of which include the production and secretion of sebum by the sebaceous glands (20, 21). Sebum, a liquid concoction of antimicrobial lipids, is composed of 28% free fatty acids, 32% triglycerides, 25% wax esters, and 11% squalene (22). Sapienic acid ($C_{16:1\Delta6}$), is the major constituent of sebum triglycerides and fatty acids, and is the primary antimicrobial fatty acid of sebum (21), which as with other unsaturated free fatty acids, appears to interfere with cell growth by altering cell permeability, uncoupling oxidative phosphorylation or by blocking electron transport (23–28). *S. aureus* is also exposed to linoleic acid ($C_{18:2}$) from nasal secretions during colonization of the anterior nares, in addition to

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substantial quantities of unsaturated triglycerides and free fatty acids within abscesses (29–31).

Apparently at odds with the documented toxicity of antimicrobial fatty acids and the occurrence of sapienic acid as the major component of sebum triglycerides, *S. aureus* produces abundant quantities of secreted glycerol ester hydrolase (lipase) enzymes (32), which function to liberate free fatty acids from triglycerides (33, 34). This raises the question as to how *S. aureus*, and in particular USA300, can colonize and persist on human skin while producing a lipase that would liberate additional toxic fatty acids from the sebum triglyceride fraction. In this context, the foundation of our present study relates to our previous observation that subinhibitory concentrations of antimicrobial fatty acids promoted the robust induction of secreted protease expression in *S. aureus* USA300, which led to maturation of a secreted lipase (35). Over a half dozen different lipases have been described in the staphylococci, including two from *S. aureus*, and the literature on these enzymes can be confusing because many are simply referred to as either lipase or Geh, for glycerol ester hydrolase. In an attempt to clarify this, a review by Rosenstein and Götz provided a naming scheme for the various lipases (34), and we have used this terminology in this study. The name *geh* has historically been used to refer to the lipase-encoding gene. To differentiate between the two lipase genes in *S. aureus*, we have named them *gehA* and *gehB*.

Here, we report on the substrate specificity and role of *S. aureus* lipase 2 (SAL2) (33, 34), encoded by the *gehB* gene (also known as *geh*), in controlling the growth of *S. aureus* in the presence of trilinolein as a model triglyceride and the functional consequence of lipase processing by the metalloprotease aureolysin (Aur).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Becton, Dickinson and Company), while *S. aureus* strains were grown in tryptic soy broth (TSB) (Becton, Dickinson and Company). All strains were grown at 37°C in an orbital shaker set at 200 rpm, unless stated otherwise. For strains carrying resistance genes, antibiotics were used as follows: chloramphenicol (10 μg/ml) and erythromycin (3 μg/ml) for growth of *S. aureus* strains and ampicillin (100 μg/ml) and kanamycin (40 μg/ml) for growth of *E. coli* strains. Solid media were supplemented with 2% (wt/vol) Bacto agar (Difco).

Strain construction. Primers used in the generation of different mutant strains are listed in Table S1 in the supplemental material. The *S. aureus* USA300 Δ*gehB* deletion mutant strain (H2660) was constructed by in-frame allelic replacement (36). Briefly, the sequences flanking the *gehB* locus (SAUSA300_0320) of USA300 FPR3757 were amplified by PCR using primers *gehB* 5′F and *gehB* 5′R to generate the upstream arm and primers *gehB* 3′F and *gehB* 3′R to generate the downstream arm. A PCR amplicon of the joined DNA fragments was recombined into the temperature-sensitive pKOR-1 vector. The resulting pKOR-1Δ*gehB* vector was first passaged through *S. aureus* RN4220 before being introduced into USA300 by electroporation. The correct deletion of codons 25 to 631 of the *gehB* gene was confirmed by PCR and DNA sequence analysis.

For complementation, the *gehB* gene and flanking regions were PCR amplified using primers Comp-*gehB*-F and Comp-*gehB*-R and cloned into the pALC2073 vector (37) using the restriction enzymes KpnI and SacI. The resulting p*gehB* vector was passaged through RN4220 and introduced into a recipient USA300 Δ*gehB* strain via electroporation, yielding the H2987 strain. The presence of the *gehB* gene in the complement strain was confirmed by PCR using primers *gehB*-int-F and *gehB*-int-R and by DNA sequence analysis. The H2988 strain was generated by introducing the plasmid pALC2073 into the USA300 Δ*gehB* strain.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
S. aureus		
USA300 LAC	Community-associated MRSA; wild-type strain, cured of resistance plasmid	35
RN4220	r_K^- m_K^+; capable of accepting foreign DNA	67
H2660	USA300 Δ*gehB*	This study
H2789	USA300 Δ*aur*::Erm; Erm^r	35
H2987	USA300 Δ*gehB* containing p*gehB*; Cm^r	This study
H2988	USA300 Δ*gehB* containing pALC2073; Cm^r	This study
RN6390 Δ*sarA*	*sarA*::Km transduced from PC1839 into RN6390	38
E. coli		
DH5α	F$^-$ ϕ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169 phoA*	Invitrogen
BL21(DE3)	*fhuA2* [*lon*] *ompT gal* (λ DE3) [*dcm*] Δ*hsdS* λ DE3 = λ sBamHIo ΔEcoRI-B *int*::(*lacI*::*PlacUV5*::T7 gene 1) *i21* Δ*nin5*	68
H2989	BL21 expressing SAL2; Km^r	This study
H2992	BL21 expressing SAL2 Ser412Ala; Km^r	This study
Plasmids		
pKOR-1	*E. coli*-*S. aureus* shuttle vector; contains $P_{xyl/tetO}$; antisense *secY* RNA expression	36
pKOR-1Δ*gehB*	pKOR-1 carrying the *gehB* deletion construct	This study
pET28a(+)	T7 expression vector carrying N-terminal His Tag/thrombin/T7 tag; Km^r	Novagen
pBC02	pET28a(+) expressing SAL2; Km^r	This study
pBC05	pET28a(+) expressing SAL2 Ser412Ala; Km^r	This study
pALC2073	*E. coli*-*S. aureus* shuttle vector; contains $P_{xyl/tet}$; Amp^r Cm^r	37
p*gehB*	pALC2073 carrying *gehB*; Amp^r Cm^r	This study

[a] Abbreviations: Erm^r, resistance to erythromycin; Cm^r, resistance to chloramphenicol; Km^r, resistance to kanamycin; Amp^r, resistance to ampicillin.

Visualization of the secreted proteome, mass spectrometry (MS), and Edman sequencing. Proteins in the cell-free culture supernatant were precipitated following a method described previously (38). Briefly, the supernatant derived from 2.5 optical density at 600 nm (OD_{600}) units of culture was mixed with an equal volume of ice-cold 20% (wt/vol) trichloroacetic acid (TCA), washed in ice-cold 70% ethanol, and then air dried and dissolved in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reducing buffer. Proteins were separated on a 4 to 12% gradient bis-Tris acrylamide gel by SDS-PAGE in 25 mM Tris-HCl–192 mM glycine–0.1% SDS running buffer, as previously described (39). After electrophoresis, gels were stained with Coomassie brilliant blue R-250 for visualization of the protein bands.

Identification of Coomassie blue-stained proteins was conducted at the London Regional Proteomics Centre at the University of Western Ontario. Protein bands were excised using an Ettan Spot Picker and processed for mass spectrometry using a Waters MASSPrep Automated Digester. Processed samples were spotted on matrix-assisted laser desorption ionization (MALDI) plates and analyzed on an Applied Biosystems

4700 Proteomics Analyzer. Data were acquired and processed using 4000 Series Explorer and Data Explorer (Applied Biosystems), and the peptide fingerprints were compared to the NCBInr database for Gram-positive bacteria, using the MASCOT search engine.

For determination of the mass of mature SAL2, purified SAL2 (10 μM) processed with 10 nM Aur for 3 h at 37°C was submitted to the Biological Mass Spectrometry Laboratory at the University of Western Ontario for analysis by electrospray ionization (ESI)-MS.

Edman sequencing of the N termini of mature forms of SAL2, taken both from USA300 culture supernatants and from Aur-treated pure proSAL2, was performed at the SPARC BioCentre at SickKids hospital in Toronto.

Construction of the *E. coli* strains expressing the recombinant forms of SAL2. For expression of recombinant SAL2, the *gehB* gene (SAUSA300_0320), without the signal peptide (first 111 bp), was amplified by PCR from the *S. aureus* strain USA300 using primers *gehB*-noSP-F and *gehB* 3′R-EcoRI containing NdeI and EcoRI restriction sites, respectively. The amplicon was digested with NdeI and EcoRI and cloned into NdeI-EcoRI-digested pET28a(+) (Novagen), which incorporates a thrombin-cleavable His_6 tag at the N terminus of the encoded recombinant protein. The resulting pBC02 was passaged into *E. coli* DH5α and then introduced into *E. coli* BL21(DE3), yielding the H2989 strain. The presence of the *gehB* gene in pBC02 was confirmed by PCR using primers *gehB*-int-F and *gehB*-int-R and by DNA sequence analysis.

The H2992 strain, expressing SAL2 Ser412Ala, was constructed by site-directed mutagenesis following the QuikChange site-directed mutagenesis protocol (Stratagene). Briefly, the entire plasmid, pBC02, was amplified using the mutagenic primers S412A and S412A_antisense, which contained 2 mismatched base pairs yielding a mutation of the serine to an alanine at the 412th amino acid residue of SAL2. Methylated, nonmutated template DNA was eliminated by enzymatic digestion with DpnI. The resulting plasmid, pBC05, was passaged through *E. coli* DH5α and then introduced into *E. coli* BL21. The presence of the *gehB* gene in pBC05 was confirmed by PCR using primers *gehB*-int-F and *gehB*-int-R, while the mutation was confirmed by DNA sequence analysis.

Purification of SAL2 and Aur. For purification of SAL2 and SAL2 Ser412Ala, *E. coli* cells were grown in LB medium containing kanamycin at 30°C. When the culture reached an OD_{600} of 0.6, isopropyl β-D-1-thiogalactopyranoside was added to a concentration of 0.5 mM. The culture was incubated for another 16 h at 25°C, after which cells were collected via centrifugation. The cell pellet was resuspended in 30 ml buffer A (10 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM imidazole). The cells were lysed using a cell disrupter (Constant Systems Inc.) at 30 lb/in^2, and 10 μM E64, a cysteine protease inhibitor, was added to the lysate. Cellular debris was first removed by centrifugation (2,500 × *g* for 15 min), followed by ultracentrifugation (255,000 × *g* for 60 min), and the soluble lysate was applied to a nickel-loaded 1-ml HisTrap column (GE Healthcare) equilibrated with buffer A. The His_6-tagged protein was eluted from the column with a gradient of 0% to 80% buffer B (10 mM Tris-HCl [pH 8.0], 300 mM NaCl, 500 mM imidazole) using an Äkta fast protein liquid chromatograph (FPLC) (GE Healthcare). Protein fractions were collected and dialyzed against 50 mM Tris, pH 8.0. Protein purity was confirmed using SDS-PAGE, and the protein was quantified using a Bradford assay.

Mass spectrometry (MS) was used to identify the different protein bands appearing on a gel upon electrophoresis of purified SAL2. Protein bands were excised using an Ettan Spot Picker and processed for MS using a Waters MASSPrep Automated Digestor. Processed samples were spotted on MALDI plates and analyzed on an Applied Biosystems 4700 Proteomics Analyzer. Data were acquired and processed using 4000 Series Explorer and Data Explorer (Applied Biosystems), and the peptide fingerprints were compared to the NCBInr database for Gram-positive bacteria, using the MASCOT search engine.

For purification of Aur, RN6390 Δ*sarA* was grown overnight, and the cell-free supernatant was collected. The proteins in the supernatant were precipitated by adding ammonium sulfate up to 85% saturation. The precipitate was then resuspended in binding buffer (20 mM Tris-HCl [pH 7.4], 5 mM $CaCl_2$) and loaded on a Sepharose-packed 5-ml HiTrap column. The protein was eluted over a gradient of 0% to 80% elution buffer (20 mM Tris-HCl [pH 7.4], 500 mM NaCl, 5 mM $CaCl_2$). Protein fractions were collected and dialyzed against 20 mM Tris (pH 7.4) with 5 mM $CaCl_2$. Protein purity was confirmed using SDS-PAGE, and the protein was quantified using a Bradford assay.

Protease activity of Aur was confirmed using fluorescein isothiocyanate (FITC)-casein substrate (Sigma). Aur was diluted in 500 μl to a final concentration of 6.25 nM and mixed with 460 μl of incubation buffer (40 mM Tris-HCl [pH 7.4], 300 mM NaCl, 20 mM $CaCl_2$, and 2 mM L-cysteine) and 50 μl of 0.2% (wt/vol) FITC-casein. EDTA was used as an Aur inhibitor. The samples were incubated at 37°C for 2 h in the dark. TCA was then added to 4% (wt/vol) to stop the reaction, and the samples were centrifuged (27,000 × *g* for 15 min) to pellet undigested casein. The supernatant was then mixed with an equal volume of 0.5 M Tris-HCl (pH 8.5), and after transfer to Optilux black clear-bottom microtiter plates (BD Falcon), fluorescence was quantified on a Biotech plate reader using excitation at 485 nm and emission at 535 nm.

Processing of SAL2 by Aur. SAL2 processing by Aur was examined by gel electrophoresis. Briefly, 50 pmol of SAL2 was first incubated at 37°C for 30 min in lipase buffer (50 mM Tris-HCl, pH 8.0) containing various amounts (0 to 50 pmol) of Aur. Reactions were stopped with the addition of SDS-PAGE reducing buffer, followed by heating for 5 min at 95°C, prior to loading on a 10% acrylamide gel. After electrophoresis, gels were stained as described above.

Lipase assay. Lipase activity was assayed with *para*-nitrophenyl palmitate (*p*NPP) or *para*-nitrophenyl butyrate (*p*NPB) substrate (Sigma) according to a method described previously (40). For assessment of lipase activity in cell-free supernatants, cultures were grown for 15 h and the supernatant was filtered across a 0.22-μm filter. Solution A (8 mM *p*NPP or *p*NPB substrate in isopropanol) was added to solution B (0.005% Triton X-100, 50 mM Tris-HCl [pH 8.0], 1 mg/ml gum arabic) at a 1:9 ratio. The culture density (OD_{600}) was determined prior to preparation of cell-free culture supernatant, and prior to assay, the supernatants were normalized to an OD_{600} of 2.0 by addition of 50 mM Tris-HCl, pH 8.0. Thereafter, 100 μl of normalized supernatant was added to 900 μl of assay buffer (solutions A and B). To measure the lipase activities of the various forms of purified SAL2, 1 nM enzyme was added to the assay buffer. To generate mature SAL2, 1 nM proSAL2 was incubated with 25 pM Aur in lipase buffer (50 mM Tris-HCl, pH 8.0) for 3 h at 37°C prior to addition of the substrate solution. Samples were incubated for 5 to 60 min at 37°C in the dark, and absorbance was quantified at 410 nm using a Varian Cary 50 spectrophotometer. *para*-Nitrophenol standards were diluted to various concentrations in assay buffer and measured, as described above, to prepare a standard curve. Absorbances obtained for each test sample were converted to amount of *para*-nitrophenol released per minute, and specific enzymatic activity was determined by accounting for the amount of protein added per sample.

Influence of triglycerides on *S. aureus* growth. For growth analyses, cells were pregrown overnight at 37°C in 5 ml TSB. A 25-ml volume of TSB, alone or supplemented with 50 μM trilinolein (TCI America), tributyrin (Sigma), triolein (Sigma), or oleic acid (Sigma), was then inoculated to achieve a starting OD_{600} equivalent of 0.01. The cultures were grown at 37°C, and the OD_{600} was measured at regular intervals. When washed cells were used, they were harvested from an overnight culture, washed three times in saline, and resuspended in fresh TSB prior to inoculating at an OD_{600} equivalent of 0.01. Where indicated, cultures inoculated with washed cells were further supplemented with an equivalent volume of sterile TSB or cell-free supernatant from isogenic cultures that differed in lipase or protease expression. Alternatively, supernatants were fractionated using centrifugal filters with a 30-kDa cutoff (Centricon), and the filtrate or retentate was added to cultures as indicated. To test the role of SAL2 in the influence of triglycerides on growth, 0.06 to 3 μg purified SAL2 or SAL2 Ser412Ala was incubated with 8.5 ng (3 pM) Aur, incubated

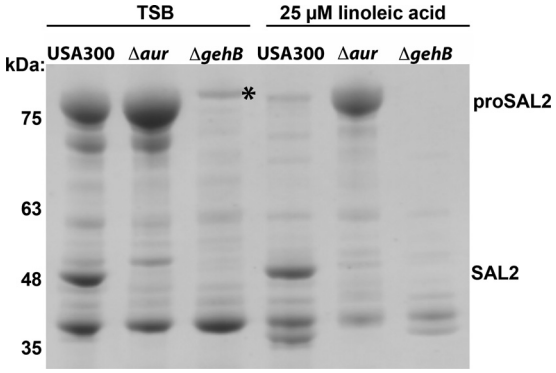

FIG 1 Induction of the staphylococcal proteolytic cascade by linoleic acid results in the Aur-dependent processing of proSAL2 to SAL2 in *S. aureus* culture supernatants. Coomassie blue-stained SDS-PAGE separation of secreted proteins from cells grown in TSB or TSB supplemented with 25 μM linoleic acid for 18 h is shown. Proteins in the culture supernatants, equivalent to 2.5 OD_{600} units, were precipitated with TCA and solubilized in SDS-PAGE reducing buffer prior to loading on a 4 to 12% bis-Tris SDS-polyacrylamide gradient gel. The experiment was repeated three times, and a representative gel is shown. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS was used to confirm the identify of proSAL2 and SAL2, as indicated, and proSAL1 (indicated by the asterisk).

at 37°C for 3 h, and added to the cultures upon inoculation. Addition of 3 pM purified Aur to the cultures was used as a negative control.

Lipid extraction and analysis by GC-MS. The substrate trilinolein was tested against 37.3 pmol of the different forms of SAL2. To obtain mature SAL2 or SAL2 Ser412Ala, 37.3 pmol of each protein were incubated in lipase buffer (50 mM Tris-HCl, pH 8.0) with 0.075 pmol of Aur for 3 h at 37°C. After incubation, trilinolein was added to a final concentration of 0.5 mM in a total 50-μl final reaction volume, and the reaction was carried out for 60 min at 37°C. Palmitic acid was added at the same time as trilinolein to each sample to serve as an internal standard for normalization. The reaction mix was acidified by addition of 200 μl of HCl (0.03 N), the fatty acids were then extracted with 1 ml of hexane, and the hexane extracts were dried under a constant stream of nitrogen gas. Dried samples were trimethylsilylated with 50 μl of pyridine and 50 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSFTA) plus 1% trimethylchlorosilane (TMS) (Sigma) for 40 min at 70°C. Released linoleic acid (as the TMS ester) was analyzed by gas chromatography (GC) on a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID) and an ion trap mass spectrometer (Varian MS 220). The GC was equipped with a pair of CP-Sil 5 CB low-bleed MS columns (WCOT silica; 30 m by 0.25 mm [inner diameter]), one in line with the FID and the other with the MS. The injector ovens were set at 250°C and the FID oven at 300°C. Samples (1 μl) were injected twice (once to each column) in splitless mode and simultaneously eluted with the following oven temperature program: the initial temperature of 80°C was held for 2 min, followed by a rapid increase (40°C per minute) to 220°C and a slower increase (15°C per minute) to 300°C. The final temperature was held for just over 4 min for a total run time of 15 min. High-purity He was used as a carrier gas at 1 ml per minute. Compounds were identified on the basis of their coelution with authentic standards and their EI-mass spectra (50 to 650 amu). Quantification was based on FID peak areas and independently derived calibration curves for each standard.

RESULTS

Processing of *S. aureus* proSAL2 to SAL2 requires Aur. The staphylococcal lipases are synthesized as prepro enzymes of approximately 75 kDa, then processed by signal peptidase during secretion, and finally further processed extracellularly by proteolytic cleavage to yield the 34- to 46-kDa mature form of the lipase.

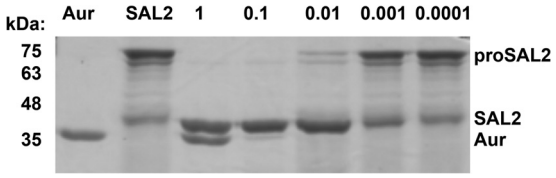

FIG 2 Processing of purified SAL2 by purified Aur. Coomassie blue-stained SDS-PAGE separation of purified proSAL2 exposed to purified Aur is shown. Lanes 1 and 2 contain 50 pmol of Aur and proSAL2, respectively. Lanes 3 to 7 contain 50 pmol of SAL2 incubated with decreasing molar ratios of Aur, as indicated, for 30 min at 37°C. The reaction was stopped with the addition of SDS-PAGE reducing buffer prior to loading. The experiment was repeated three times, and a representative gel is shown.

A secreted lipase of *Staphylococcus hyicus* was first shown to be processed by a secreted metalloprotease (41), and we have similarly noted that SAL2 of *S. aureus* accumulates in the culture supernatant in the 72-kDa precursor form when Aur function is inactivated (35), consistent with earlier reports that SAL2 processing could be prevented by supplementing an *S. aureus* culture with either metalloprotease or cysteine protease inhibitors (42). While studies have reported that the precursor and mature forms were fully active (34, 41, 42), these conclusions were based on zymogram assays of the proteins after initial separation by SDS-PAGE. However, this is not a good means of differentiating between the activities of mature and precursor isoforms. Notably, although tissue matrix metalloproteases are expressed as inactive precursors that must undergo proteolytic activation, the precursor and mature forms are both fully active in gelatin zymogram assays, because the initial SDS-PAGE exposes the active site, allowing the precursor to exhibit activity on subsequent renaturation in the acrylamide gel (43). Therefore, our first goal was to assess the specificity of proSAL2 processing by Aur and then to evaluate the activities of proSAL2 and mature SAL2 under optimal conditions in aqueous buffers.

As reported by us previously and by others, proSAL2 was among the most abundant secreted proteins when *S. aureus* USA300 was grown in TSB (Fig. 1), and the mature form of approximately 48 kDa was also evident. We constructed a *gehB* deletion in USA300 and showed that this strain produced neither the pro nor the mature form of SAL2 in the culture supernatant (Fig. 1). In USA300 Δ*aur*, the 72-kDa precursor was more abundant, and the mature form was not evident. When USA300 was cultured in TSB containing 25 μM linoleic acid, proSAL2 was no longer evident, but mature SAL2 remained as a predominant secreted protein. Conversely, when USA300Δ*aur* was grown with 25 μM linoleic acid, proSAL2 was abundant in the supernatant, but mature SAL2 was not evident (Fig. 1). Cumulatively, these data confirm that Aur is required for processing of proSAL2, which is accelerated when cultures are supplemented with linoleic acid, due to induction of *aur* expression.

To evaluate the specificity and sensitivity of this processing, we next conducted assays with purified proteins. Aur was purified from the supernatant of *S. aureus* RN6390 Δ*sarA*, which overexpresses Aur in the culture supernatant, since *sarA* is a repressor of protease expression (44). Although Aur was readily purified from *S. aureus* culture supernatants and was stable (Fig. 2), recombinant proSAL2 purified from *E. coli* cytoplasm yielded a predominant approximately 75-kDa protein and numerous smaller polypeptides, which were confirmed by mass spectrometry to be

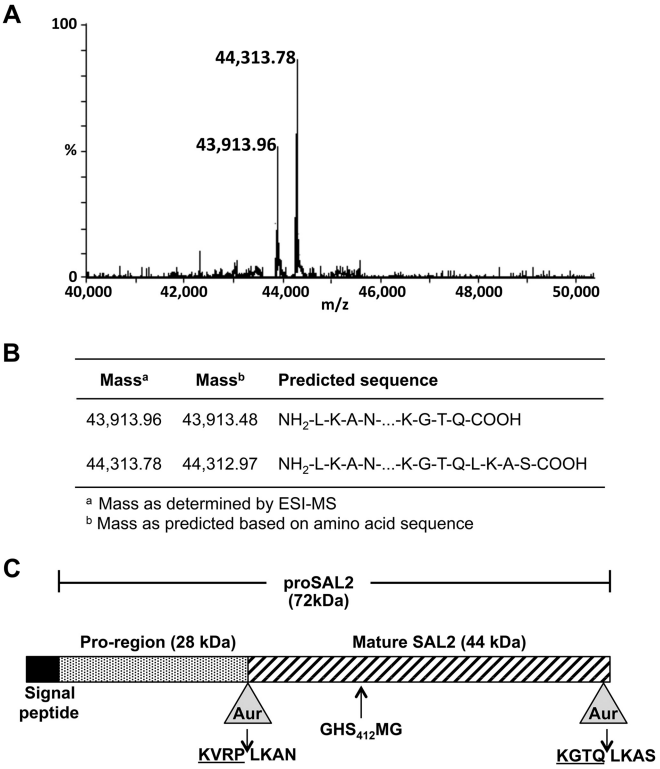

FIG 3 Processing of SAL2 by Aur yields two major peptide species. (A) Mass spectral data of SAL2. proSAL2 (10 μM) was first incubated with 10 nM Aur at 37°C for 3 h to ensure complete processing to SAL2 and then dialyzed in water at 4°C for 24 h prior to analysis by ESI-MS. (B) Predicted sequences of the two major peptide species of SAL2 released upon processing by Aur. (C) Diagram of the SAL2 protein. The triangles indicate the Aur processing sites, and the processing sequences are listed below each site. The arrow indicates Ser412, which is an essential component of the catalytic triad in SAL2.

fragments of SAL2 (data not shown). Nevertheless, when this heterogeneous mixture was incubated with Aur, a single approximately 42-kDa polypeptide was the major product observed after a 30-min incubation at 37°C (Fig. 2). Aur was highly active on proSAL2, since even at an Aur/proSAL2 molar ratio approaching 1:1,000, proSAL2 was readily processed within 30 min, and after 2 h of incubation, all proSAL2 was processed to SAL2 at a molar ratio of 1:10,000 (data not shown). Overall, our data clearly demonstrate that processing of SAL2 from its pro form to its mature form is both dependent on and highly sensitive to the metalloprotease Aur.

Mass spectrometry analysis of the Aur-treated recombinant proSAL2 yielded identification of two major species (Fig. 3). The larger of these had a mass of 44,313.78 Da, corresponding to the mass of a predicted fragment beginning at ^{294}LKAN and ending at the C terminus of the protein. This processing site, at RP ↓ ^{294}LKAN, differs by two residues from the processing site predicted based on N-terminal sequencing of SAL2 protein purified from *S. aureus* culture supernatant, RPLK ↓ ^{296}AN (42). The second species, with a mass of 43,913.96 Da, corresponded to a polypeptide with the same N-terminal sequence but lacking a C-terminal tetrapeptide, ^{687}LKAS. Both of these sites are in agreement with leucine as the preferred P1′ amino acid in known Aur cleavage sites, as identified in the MEROPS peptidase database (http://merops.sanger.ac.uk/index.shtml).

Edman degradation sequencing was performed on mature SAL2 from USA300 culture supernatants and from *in vitro* reaction mixtures of proSAL2 incubated with Aur. These analyses confirmed Leu294 to be the N-terminal amino acid in mature SAL2 from both culture supernatants and *in vitro* processing reactions.

SAL2 is required for lipase activity on short- and long-chain fatty acid substrates. *S. aureus* has two lipase genes, which in USA300 are SAUSA300_2603 (*gehA*, encoding SAL1) and SAUSA300_0320 (*gehB*, encoding SAL2). Of the products, SAL1 has been best characterized at the molecular level, which includes cloning and expression of the structural gene, indicating that SAL1 is optimally active at pH 6.0 and primarily specific for triacylglycerols of short-chain fatty acids (45, 46). However, a similar level of molecular detail for SAL2 is lacking. In one study, a lipase purified from *S. aureus* strain FN37 hydrolyzed monoacyl-, diacyl-, and triacylglycerol esters of oleic acid (33, 47), and although its identity was not known at the time, its reported amino acid composition appears to closely match the predicted composition of mature SAL2. Similarly, Lee and Iandolo found that a temperate phage in *S. aureus* strain PS54 could inactivate *gehB*, leading to an inability of *S. aureus* supernatants to hydrolyze triglycerides in egg yolk (48, 49), which contains large amounts of long-chain fatty acids, including palmitic, oleic, and linoleic acids (50). However, neither the biochemical properties of SAL2 nor its contribution to total lipase activity in *S. aureus* culture supernatant has been addressed at the molecular level.

To address this, we first conducted assays with chromogenic substrates (40), using *p*NPP as a representative saturated ($C_{16:0}$) long-chain fatty acid ester and *p*NPB as a short-chain ($C_{4:0}$) fatty acid ester. We could readily detect activity on both substrates, using stationary-phase culture supernatant of USA300 grown in TSB (Fig. 4A and B), although the activity toward *p*NPP was approximately double that which was measured using *p*NPB. Strikingly, supernatant from USA300 Δ*gehB* exhibited no activity on the *p*NPP substrate, and there was also a major significant reduction in activity toward *p*NPB. Moreover, activity toward both substrates was restored beyond wild-type (WT) levels in USA300 Δ*gehB* carrying plasmid p*gehB*, which carries a copy of the *gehB* gene. Once again, the activity toward *p*NPP was approximately 2- to 3-fold greater than that measured using *p*NPB. Cumulatively, these observations indicate that SAL2 can hydrolyze both long- and short-chain fatty acid esters, but with a preference for the longer-chain fatty acid substrates.

To corroborate these findings, purified proSAL2 and mature SAL2 generated by *in vitro* processing of proSAL2 with Aur were incubated with both *p*NPB and *p*NPP. SAL2 had high enzymatic activity toward *p*NPP, independent of whether the protein was in its precursor or mature form, indicating that proSAL2 is no less able to cleave *p*NPP than the mature form of the enzyme (Fig. 4C). proSAL2 and SAL2 also equally hydrolyzed *p*NPB, albeit at a lower efficiency than when *p*NPP was used as the substrate (Fig. 4D). Together, these results suggest that *S. aureus* SAL2 is a lipase with preferred activity on esters of long-chain fatty acids but which can also hydrolyze esters of short-chain fatty acids. Moreover, although *S. aureus* USA300 expresses SAL1 (Fig. 1), an enzyme previously shown to have activity on short-chain fatty acid substrates (45, 46), our data indicate that under our culture conditions, SAL2 is primarily responsible for lipase activity toward both short- and long-chain fatty acid substrates in culture supernatant.

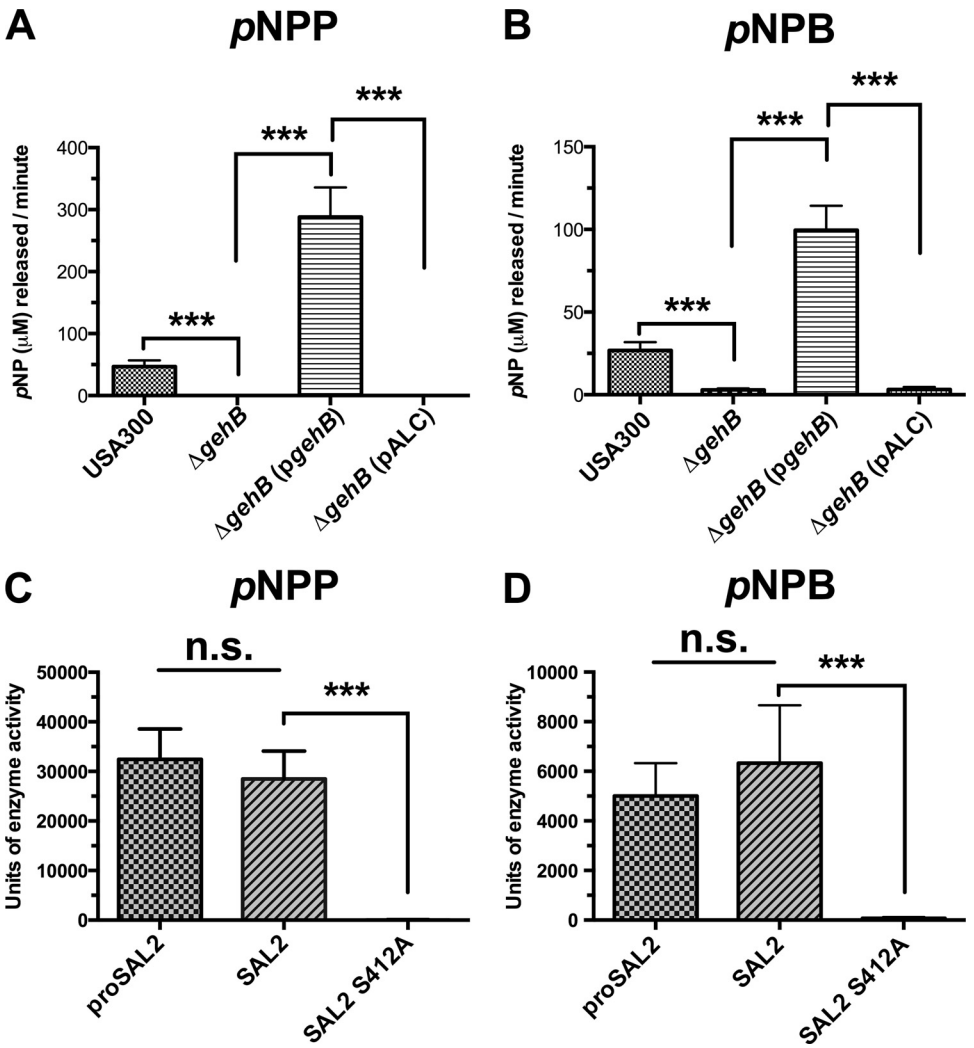

FIG 4 SAL2 is required for lipase activity on short-chain and long-chain fatty acids. (A and B) Lipase activity was assayed on spent culture supernatants from cells grown in TSB for 15 h. Equivalents of 0.2 OD_{600} unit of culture supernatant were incubated with either *para*-nitrophenyl palmitate (*p*NPP) (A) or *para*-nitrophenyl butyrate (*p*NPB) (B) for 5 to 60 min at 37°C. Release of *para*-nitrophenol (*p*NP) was measured by spectrophotometry at 410 nm, and the lipase activity was calculated as the amount of *p*NP released per minute (see Materials and Methods). (C and D) Lipase activity was assayed on various forms of purified SAL2 (1 nM) using either *p*NPP (C) or *p*NPB (D), as described in Materials and Methods. All enzymatic assays were measured on three biological and three technical replicates. The data are plotted as average ± standard deviation. Statistical analysis was performed with Student's *t* test (***, $P < 0.0001$; n.s., not significant). Δ*gehB* (p*gehB*) represents the USA300 Δ*gehB* strain containing the complementing p*gehB*. Δ*sal2* (pALC) represents the USA300 Δ*gehB* strain containing the empty pALC2073 vector. One unit of enzyme activity refers to the amount of enzyme that releases 1 μmol of *para*-nitrophenol per minute.

Identification of an active-site serine in *S. aureus* SAL2. Other lipases, including pancreatic lipase, human lipoprotein lipase, fungal lipases, and bacterial lipases, harbor a common catalytic triad consisting of Ser, Asp or Glu, and His (51, 52). The catalytic Ser is usually found within a conserved Gly-Xxx-Ser-Xxx-Gly motif (53). Therefore, based on homology with other staphylococcal lipases, the predicted catalytic triad in SAL2 is comprised of Ser412, Asp603, and His645, and the Ser412 is within the motif ^{410}Gly-His-Ser-Met-^{414}Gly (Fig. 3C). In order to generate a catalytically inactive SAL2 enzyme, we thus chose to construct and purify SAL2 containing a Ser412Ala substitution. As shown in Fig. 4C and D, the SAL2 Ser412Ala variant had no activity toward either the *p*NPP or *p*NPB substrate, confirming Ser412 as a part of the essential catalytic triad of the enzyme.

SAL2 hydrolyzes trilinolein to linoleic acid. The *p*NPP-based lipase assay is a valuable tool to identify lipase activity, but it does not utilize a natural substrate. We therefore proceeded to test whether SAL2 had activity on long-chain triglycerides such as trilinolein, which is a naturally occurring triacylglycerol found in oils from plants, including sunflower, sesame, linseed, and safflower (54). For these assays, we used GC-MS because it could readily detect linoleic acid and palmitic acid standards (Fig. 5A and B, respectively). GC-MS is not capable of detecting trilinolein, and the GC-MS analysis of trilinolein indicated that it did not contain detectable quantities of linoleic acid as a breakdown product (Fig. 5C). When we incubated mature (i.e., Aur-treated) SAL2 in the presence of trilinolein, however, we could clearly measure the release of linoleic acid (Fig. 5D), whereas no linoleic acid was released when trilinolein was incubated with the Ser412Ala variant of SAL2 (Fig. 5E). As another control, we demonstrated that Aur

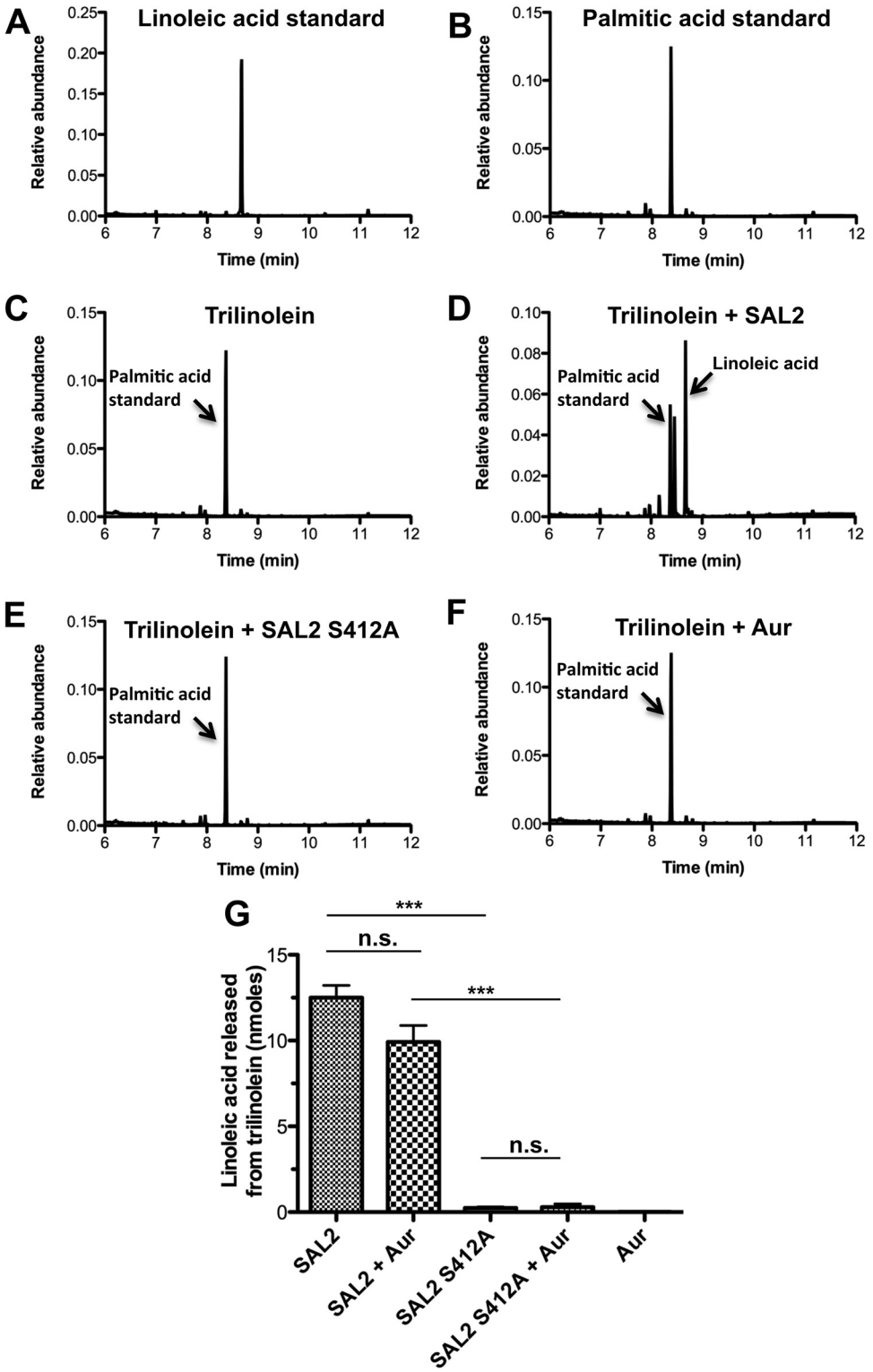

FIG 5 SAL2 hydrolyzes trilinolein to linoleic acid. (A and B) Standards for linoleic acid (A) and palmitic acid (B) run at 8.8 and 8.4 min, respectively. (C to F) Detection of the relative abundance of linoleic acid (peak at 8.8 min) released upon hydrolysis of trilinolein in the presence of no addition (C), SAL2 (D), SAL2 Ser412Ala (E), or Aur (F). Reactions for panels D and E were carried out by incubating 37.3 pmol of protein with 0.5 mM trilinolein for 1 h at 37°C. Fatty acids were then extracted and derivatized, and the amount of linoleic acid was measured by GC-MS (see Materials and Methods for details). Palmitic acid was used as a normalizing standard (peak at 8.4 min). Since small amounts of Aur were used to produce the SAL2 that was used in these assays, we showed that Aur, even at significantly higher concentrations, had no hydrolytic activity on trilinolein (F). (G) Total amount of linoleic acid released from trilinolein as extrapolated from a linoleic acid standard curve. The experiments were repeated with three technical replicates on a minimum of three biological replicates, and the data are plotted as average ± standard deviation. Statistical analysis was performed with Student's *t* test (***, $P < 0.0001$).

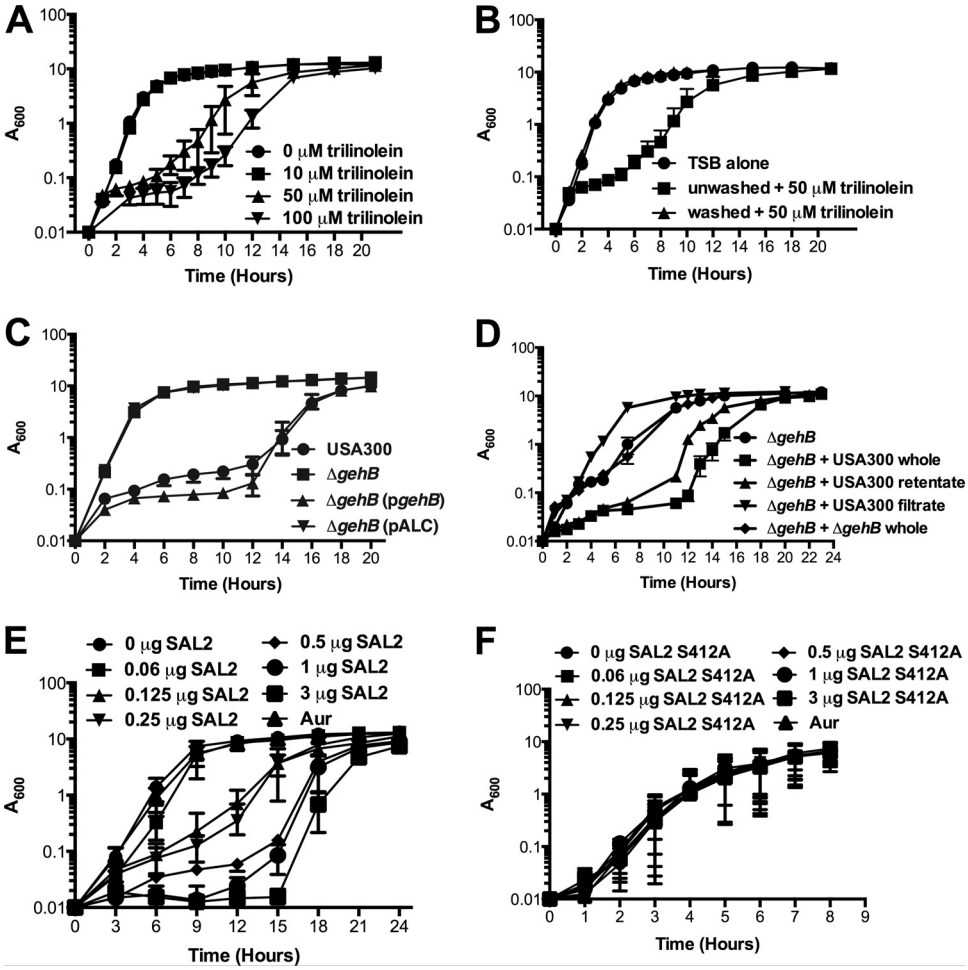

FIG 6 SAL2 impairs growth of *S. aureus* in the presence of trilinolein. (A) Growth of USA300 in TSB alone or supplemented with various concentrations of trilinolein, as indicated. (B) Precultured USA300 cells (unwashed or washed three times with saline, as indicated, prior to subculture) were grown in TSB alone or TSB supplemented with 50 μM trilinolein. (C) Growth of various strains in TSB supplemented with 50 μM trilinolein. Δ*gehB* (p*gehB*) refers to the USA300 Δ*gehB* strain containing the complementing p*gehB*, while Δ*gehB* (pALC) represents the USA300 Δ*gehB* strain containing the empty pALC2073. (D) Growth of USA300 Δ*gehB* in 25 ml TSB supplemented with 50 μM trilinolein and 100 μl of culture supernatant (whole, retentate, or filtrate) from USA300 or USA300 Δ*gehB*, as indicated. Whole refers to the whole supernatant, retentate refers to the supernatant that was retained within the 30-kDa centrifugal column upon fractionation of the supernatant, and filtrate refers to the supernatant that went through the 30-kDa centrifugal filter. (E and F) Growth of USA300 Δ*gehB* in 25 ml TSB supplemented with 50 μM trilinolein and increasing concentration of purified Aur-processed SAL2, SAL2 Ser412Ala, or Aur, as indicated. For all panels, cultures were inoculated to a starting OD_{600} of 0.01, and growth was measured by optical density at 600 nm. All growth curves were repeated a minimum of three times. The data are plotted as average ± standard deviation.

itself was incapable of generating linoleic acid from trilinolein (Fig. 5F). The GC-MS analyses allowed us to quantitate the amount of linoleic acid released from trilinolein. Similar to the results from the *p*NPP and *p*NPB assays, proSAL2 and SAL2 both released similar amounts of linoleic acid (12.5 and 9.9 nmol, respectively), while both the mature and pro forms of the SAL2 Ser412Ala variant had virtually undetectable activity (Fig. 5G).

Trilinolein inhibits growth of USA300 in a *gehB*-dependent manner. As demonstrated above, SAL2 is an extracellular lipase that is capable of hydrolyzing long-chain fatty acid-containing triglycerides, such as trilinolein. The activity of SAL2 on trilinolein, however, results in the release of linoleic acid, an unsaturated free fatty acid that is growth inhibitory to *S. aureus* at concentrations at or above 50 μM (35). Therefore, to evaluate the consequence of SAL2 activity on trilinolein for the growth of *S. aureus*, we assessed the growth of *S. aureus* USA300 in TSB supplemented with various concentrations of trilinolein compared to that in TSB alone. We took cells from an overnight culture grown in TSB and subcultured them to an OD_{600} equivalent of 0.01 into flasks containing 25 ml fresh TSB or TSB containing 10 to 100 μM trilinolein (cells were diluted approximately 1,000-fold from the overnight culture). As expected, USA300 reached stationary phase by approximately 6 h when grown in TSB (Fig. 6A). In contrast, growth was significantly delayed in the presence of 50 μM trilinolein, and even more so in the presence of 100 μM trilinolein, while the presence of 10 μM trilinolein did not result in any delay in growth (Fig. 6A). The almost immediate and significant delay in growth in the presence of trilinolein was interesting, because it suggested that even a trace amount of SAL2 in the inoculum from the overnight culture was sufficient to rapidly generate inhibitory concentrations of linoleic acid in 25 ml of culture medium.

This assumption was confirmed through a number of indepen-

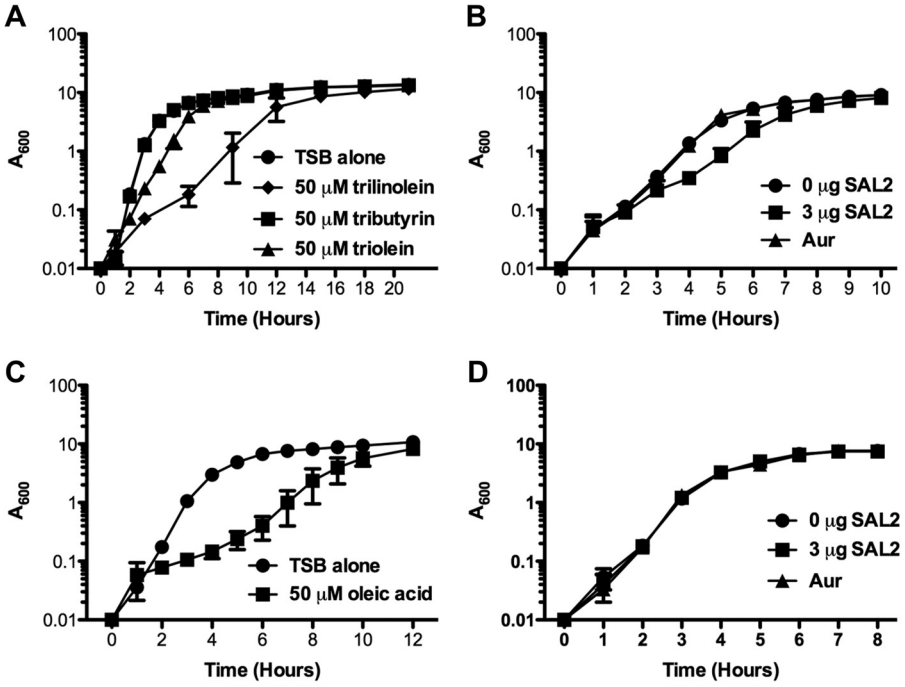

FIG 7 SAL2 impairs growth of *S. aureus* in the presence of triolein but not tributyrin. (A) Growth of USA300 in TSB alone or TSB supplemented with one of three different triglycerides, as indicated. (B) Growth of USA300 Δ*gehB* in 25 ml TSB supplemented with 50 μM triolein with or without addition of purified SAL2 or Aur, as indicated. (C) Growth of USA300 in TSB alone or TSB supplemented with 50 μM oleic acid. (D) Growth of USA300 Δ*gehB* in 25 ml TSB supplemented with 50 μM tributyrin with or without addition of purified SAL2 or Aur, as indicated. For all panels, cultures were inoculated to a starting OD_{600} of 0.01, and growth was measured by optical density at 600 nm. All growth curves were repeated a minimum of three times. The data are plotted as average ± standard deviation.

dent approaches. First, when cells from the stationary-phase inoculum were washed three times in saline prior to subculturing into TSB containing 50 μM trilinolein, growth was initiated without delay (Fig. 6B). Furthermore, growth of USA300 Δ*gehB* was unimpeded in the presence of 50 μM trilinolein, and the growth delay was restored when USA300 Δ*gehB* was complemented with a plasmid carrying *gehB* but not the empty vector (Fig. 6C). Third, we fractionated the cell-free culture supernatant from an overnight culture, by passage through a 30-kDa-cutoff Centricon filter device, prior to it being used to supplement a culture of USA300 Δ*gehB* containing 50 μM trilinolein. In these experiments, the fraction that was retained on the filter promoted a significant delay in growth, whereas the filtrate fraction that passed through the membrane did not promote a growth delay. Finally, addition of whole culture supernatant from USA300 Δ*gehB* did not have any effect on the growth of USA300 Δ*gehB* in TSB containing trilinolein (Fig. 6D). Cumulatively, these data support the contention that trace amounts of SAL2, transferred into a fresh culture from a stationary-phase inoculum, are sufficient to quickly liberate growth-inhibitory concentrations of linoleic acid in medium containing trilinolein substrate.

To elaborate on this, we next queried whether the growth delay in the presence of trilinolein was dependent on SAL2 enzymatic activity and, if so, whether the effect of SAL2 on trilinolein in modulating the growth of *S. aureus* was titratable. To resolve this, USA300 Δ*gehB* was grown in TSB containing 50 μM trilinolein, which was further supplemented at the time of inoculation with a range of concentrations of pure SAL2. As shown in Fig. 6E, the duration of the growth delay was increased with the addition of increasing concentrations of SAL2 (between 0.06 and 3 μg SAL2 added to a 25-ml culture). In contrast, even the highest concentration of SAL2 Ser412Ala added did not result in delayed growth (Fig. 6F). This observed effect was specific to cultures containing trilinolein, since SAL2, when added to USA300 Δ*gehB* cultures that did not contain trilinolein, resulted in no growth defect (data not shown). Cumulatively, our observations support the hypothesis that the growth delay observed in the presence of trilinolein was a result of SAL2-dependent liberation of growth-inhibitory concentrations of linoleic acid.

Triolein, but not tributyrin, slows growth of USA300 in a SAL2-dependent manner. Given that trilinolein inhibited the growth of *S. aureus*, we tested whether other triglycerides would have similar effects. Triolein, a triglyceride composed of glycerol and three molecules of oleic acid ($C_{18:1}$ *cis*-9) was evaluated first. Triolein is similar to trilinolein in the sense that the chains of the fatty acids esterified to glycerol are the same length (C_{18}), yet in trilinolein the fatty acid chains are diunsaturated ($C_{18:2}$), while they are monounsaturated ($C_{18:1}$) in triolein. A significant delay in growth was observed in the presence of triolein compared to when the cells were grown in TSB alone, although the delay was reproducibly not as severe as when the cells were grown in the presence of trilinolein (Fig. 7A). This is consistent with work by ourselves and others where it was shown that oleic acid is less toxic than linoleic acid (35, 55). To confirm that the triolein-dependent growth delay was due to *gehB* function, we cultured USA300 Δ*gehB* in TSB containing triolein, with or without exogenous SAL2 supplement, and again observed that exogenous SAL2 delayed the growth of USA300 Δ*gehB* (Fig. 7B). We confirmed that

the observed growth delay was dependent on the lipase activity of SAL2, which would release oleic acid from triolein, as demonstrated by a similar growth delay for USA300 cultured in the presence of oleic acid (Fig. 7C). No growth delays were detected when USA300 (Fig. 7A) or USA300 Δ*gehB* with added purified SAL2 (Fig. 7D) was grown in TSB supplemented with tributyrin, a short-chain triglyceride composed of glycerol and three molecules of butyric acid ($C_{4:0}$), which is not inhibitory to *S. aureus* (55). Overall, these data indicate that SAL2 hydrolyzes triglycerides and that the downstream effect of the release of free fatty acids on the growth of *S. aureus* is dependent on the structure of the free fatty acid (e.g., long-chain versus short-chain or saturated versus unsaturated).

DISCUSSION

In previous work, SAL2 (also known as Geh [glycerol ester hydrolase]) was identified as one of just seven secreted proteins that was universally produced by 63 diverse strains of *S. aureus* (32), and as such, it is unlikely that *S. aureus* would have evolved to maintain abundant production of SAL2 if this did not confer a substantial benefit to the bacterium. This is especially true of the epidemic USA300 strain of CA-MRSA, which is noted for its efficient transmission in the community and, as noted with many other strains of *S. aureus*, exhibits abundant *in vitro* production of SAL2 (35, 56). Therefore, the goals of our study were to determine the biochemical properties of SAL2, its contribution to the total lipase activity of *S. aureus*, the functional consequences of its processing by the metalloprotease Aur, and its effect on growth of *S. aureus* in the presence of triglyceride substrates that contain antimicrobial fatty acids, as would be encountered on the skin as a component of sebum or within abscessed tissue. To summarize our main findings, under our culture conditions, SAL2 exclusively accounted for the majority of esterase activity in USA300 culture supernatant toward both short-chain and long-chain fatty acid substrates, but exhibited significantly higher activity on long-chain substrates, and was able to quickly liberate growth-inhibitory levels of linoleic acid and oleic acid from trilinolein and triolein substrates, respectively. However, while the precursor form of the enzyme was extremely sensitive to processing by Aur, we were not able to identify any functional consequences specific to enzyme activity.

With respect to the latter conclusion, our interest in conducting this study was, in part, linked to our previous finding that antimicrobial fatty acids induce the expression of secreted proteases comprising the staphylococcal proteolytic cascade, including the metalloprotease Aur (35), which in addition to having an essential role in activation of the SspA serine protease (38) was also observed to promote conversion of the SAL2 precursor into a mature lipase (35). As with SspA, the SAL2 precursor is produced with an N-terminal propeptide (57), and it was therefore a reasonable assumption that processing of proSAL2 by Aur might serve to modulate its activity on triglyceride substrates. Accordingly, we observed that proSAL2 was extremely sensitive to processing by Aur, at RP ↓ ^{294}LKAN and a secondary site, TQ ↓ ^{687}LKAS. However, while both of these sites resemble the initial site that is processed during Aur-dependent activation of the SspA serine protease, at GN ↓ ^{58}LKPL within the N-terminal propeptide (38), our assays with both *p*NPP and trilinolein substrates were unable to establish any significant difference in the activities of the precursor and mature form of SAL2, at least under the conditions we tested and with the substrates that we used in this study. The high sensitivity and selectivity of proSAL2 processing by Aur remain at odds with an apparent lack of functional significance. Although we cannot conclude that Aur processing of proSAL2 is dispensable for all of its potential activities, the most notable of which may be in the *in vivo* environment, we can conclude that it is not required for the activities we have observed in this study. This is derived from our observations that an Aur mutant demonstrated a growth delay similar to that of WT USA300 in the presence of trilinolein (data not shown) and that supernatant from the Aur mutant had an overall lipase activity that was not statistically different from that of WT USA300 (data not shown).

Our biochemical characterization of SAL2 has helped to resolve its properties and substrate specificity in relation to a number of previous reports that have characterized staphylococcal lipases. Although we have shown that SAL2 can hydrolyze triglycerides of short-chain fatty acids, i.e., tributyrin, our data support the contention that its preferred substrates are triglyceride-containing long-chain fatty acids, as evident from its higher specific activity on *p*NPP substrate than on *p*NPB. Previous work indicated that *S. aureus* lipase was more active on triglycerides of short-chain fatty acids (46), but this was based on Lip1 (SAL1) activity (45), while here we examined SAL2. Therefore, although the mature SAL2 (SAUSA300_0320) and SAL1 (SAUSA300_2603) lipases share 57% amino acid identity, they have significant differences in substrate specificity, and SAL1 is optimally active at pH 6.0, while SAL2 exhibits negligible activity at acidic pH (see Fig. S1 in the supplemental material). However, even when assayed at acidic pH, which is optimal for SAL1 activity, there was negligible lipase activity in the culture supernatant of USA300 Δ*gehB* (Fig. S1). Therefore, under the growth conditions used in this study and consistent with its abundant expression, SAL2 is the predominant secreted lipase in *S. aureus* USA300, while SAL1, which is specific for esters of short-chain fatty acids, does not appear to have a significant contribution to total lipase activity, despite the fact that we can detect SAL1 in culture supernatants of USA300 Δ*gehB* (Fig. 1).

Unique to our study, we evaluated how SAL2 function would affect growth of *S. aureus* in the presence of exogenous triglycerides. We demonstrated that USA300 exhibited an extended lag phase when grown in the presence of trilinolein, similar to that previously observed during growth in the presence of linoleic acid (35). Although trilinolein is not a typical triglyceride that would be found in the *S. aureus* habitat (e.g., in an abscess), triglycerides that *S. aureus* is exposed to do nonetheless incorporate linoleic acid. We chose to use this particular triglyceride since we could control the identity of the fatty acid released and had preexisting knowledge as to its antimicrobial effects on *S. aureus*. From our data, we conclude that the delay in growth is a result of SAL2 hydrolyzing trilinolein to linoleic acid, and, remarkably, we demonstrated that a 20-μl inoculum from an overnight culture contained sufficient SAL2 to generate growth-inhibitory linoleic acid when transferred into 25 ml of TSB containing 50 μM trilinolein. These observations support a potent ability of SAL2 to liberate long-chain fatty acids from triglycerides, consistent with the work of Rollof et al., who characterized a lipase from *S. aureus* strain FN37 which, as with SAL2 in this study, exhibited optimal activity at pH 8.0 (33). The enzyme exhibited extremely rapid hydrolysis of trioleoylglycerol, with over 30% conversion to glycerol after 2 min of incubation and 95% conversion after 12 min (47). Although this was reported to occur without any significant accu-

mulation of partial glycerides, a different study demonstrated that *S. aureus* lipase SAL3 produced 1,3-diolein and lesser amounts of 1,2- or 2,3-diolein as the initial products of trioleoylglycerol hydrolysis, suggesting a preference for initial processing at the *sn*2 position. This lipase was designated SAL3, although it is evident from the primers that were employed for cloning of the gene (58, 59) that it corresponds to SAL2 (SAUSA300_0320) described in this study. Nonetheless, both studies demonstrated hydrolysis of fatty acid esters at all three positions of the triacylglycerol substrate, suggesting that SAL2 can achieve complete conversion of triacylglycerol into glycerol and free fatty acids. The secretion of SAL2 into an environment containing triglycerides is paradoxical if it results in the release of growth-inhibitory free fatty acids, adding to the already existing pool of these compounds on the skin or in a wound environment. Therefore, the question arises as to why *S. aureus* produces lipases. Some bacteria have the metabolic capacity to generate energy through β-oxidation of fatty acids, but *S. aureus* does not have this capacity, and exogenous fatty acids are exclusively incorporated into membrane phospholipid (25, 55, 60). *S. aureus* also does not have the capacity to synthesize unsaturated fatty acids, and membrane fluidity is therefore maintained by the production of branched-chain fatty acids, of which anteiso-pentadecanoic acid is the major fatty acid in *S. aureus* phospholipid (55, 60, 61). The *de novo* synthesis of branched-chain fatty acids begins with the degradation of branched-chain amino acids (BCAA) (Leu, Ile, and Val) (61), and an important role for BCAA in signaling the metabolic status of the cell through the CodY transcriptional regulator has recently been documented (62). In this context, although *S. aureus* appears to have all of the necessary genes for synthesis of BCAA, it requires exogenous leucine and valine for optimal growth (62–64). Therefore, it is reasonable to postulate that SAL2 functions to provide host-derived unsaturated fatty acids for incorporation into membrane phospholipid, thereby reducing the metabolic draw on available BCAA, some of which would otherwise need to be degraded to satisfy the requirement for synthesis of branched-chain fatty acids and maintenance of membrane fluidity.

However, this contention is not supported by published studies. First, although some bacteria cease *de novo* synthesis of fatty acids when provided with an exogenous supply (60), this does not occur in *S. aureus*, where synthesis of branched-chain fatty acids continues unabated in the presence of exogenous unsaturated free fatty acid, and under these conditions, incorporation of branched-chain fatty acid into membrane phospholipid is reduced but not eliminated (55, 60, 65). Therefore, from a standpoint of nutritional status, there appears to be no tangible benefit to *S. aureus* in production of SAL2 as a means of providing host-derived fatty acids, either as a source of energy or for incorporation into membrane phospholipid. This conclusion is further inherent in the potent activity of SAL2, as noted in this study and previously by others, who commented that the lack of positional specificity and substrate selectivity, combined with the potency and copious production of the enzyme, would likely liberate quantities of fatty acid that far exceed the nutritional requirements of the bacterium (47), all of which point toward alternate functions for SAL2.

In this context, given the evolutionary conservation of abundant SAL2 production by *S. aureus* (32), we postulate that a contribution to colonization and persistence on human skin is still likely to be the most plausible role. Although one study has reported a role for SAL2 in virulence using a murine peritoneal abscess model (66), it was conducted with *S. aureus* RN4220, which has been exposed to chemical mutagenesis (67). We expect that definitive evaluation of SAL2 function in the in *vivo* persistence and virulence of *S. aureus* will require a number of different models, including persistence on skin, subcutaneous abscess, and bacteremia, and studies are in progress to evaluate both SAL1 and SAL2, alone and in combination, from which we hope to obtain a full understanding of the significance of these enzymes.

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